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Role of multidrug resistance-associated protein 1 in airway epithelium

van der Deen, Margaretha

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CHAPTER TWO

ATP-binding cassette (ABC) transporters in normal and pathological lung

**Margaretha van der Deen¹, Elisabeth G.E. de Vries¹, Wim Timens³,
Rik J. Scheper⁴, Hetty Timmer-Bosscha¹ and Dirkje S. Postma²**

Department of Medical Oncology¹, Department of Pulmonary Medicine², Department of Pathology and Laboratory Medicine³, University Medical Center Groningen, The Netherlands. Department of Pathology⁴, Free University Medical Center, Amsterdam, The Netherlands



Abstract

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that can transport a wide variety of substrates across biological membranes in an energy-dependent manner. Many ABC transporters such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) are highly expressed in bronchial epithelium. This review aims to give new insights in the possible functions of ABC molecules in the lung in view of their expression in different cell types. Furthermore, their role in protection against noxious compounds, e.g. air pollutants and cigarette smoke components, will be discussed as well as the (mal)function in normal and pathological lung. Several pulmonary drugs are substrates for ABC transporters and therefore, the delivery of these drugs to the site of action may be highly dependent on the presence and activity of many ABC transporters in several cell types. Three ABC transporters are known to play an important role in lung functioning. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene can cause cystic fibrosis, and mutations in *ABCA1* and *ABCA3* are responsible for respectively Tangier disease and fatal surfactant deficiency. The role of altered function of ABC transporters in highly prevalent pulmonary diseases such as asthma or chronic obstructive pulmonary disease (COPD) have hardly been investigated so far. We especially focused on polymorphisms, knock-out mice models and *in vitro* results of pulmonary research. Insight in the function of ABC transporters in the lung may open new ways to facilitate treatment of lung diseases.

Introduction

The prime role of the airways (trachea, bronchi, bronchioles and terminal bronchioles) is to conduct air into and out of the lung and to form a first line of defence against undesired constituents of inhaled air. The airways are continuously exposed to pathogens, irritants, pollutants and agents that produce oxidative stress and therefore, the composition of the respiratory tract surface is very important. The upper airways contain specialised cell types such as ciliated cells and mucous secreting goblet cells. The lower conducting airways (respiratory bronchioles, alveolar ducts and alveolar sacs) participate in gas exchange by diffusion. The alveolar epithelial surface comprises essentially two cell types, the alveolar epithelial type I cell and the cuboidal alveolar epithelial type II cell. Type I cells flatten out and in this way constitute approximately 95% of the total alveolar surface, whereas type II cells are more numerous and produce surfactant [1].

ABC (ATP-binding cassette) transporters are a family of transmembrane proteins that can transport a wide variety of substrates across biological membranes in an energy-dependent manner. They are phylogenetically classified in seven distinct subfamilies of transporters (ABCA to ABCG), which are again divided into subgroups. To date, 48 ABC transporters have been detected in the human body [2, 3]. Overexpression of ABC transporters such as P-glycoprotein (P-gp) and the multidrug resistance-associated protein 1 (MRP1) were initially detected in tumour cell lines. Their overexpression is associated with increased efflux of chemotherapeutic drugs such as anthracyclines, epipodophyllotoxins and vinca-alkaloids, and this can result in so-called multidrug resistance (MDR). Many MDR proteins can act as drug efflux pumps, resulting in decreased intracellular concentrations of toxic compounds at the site of action. MRP1 and P-gp expression in the lung have especially been studied in the context of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Currently we know that ABC transporters are present in virtually every cell of all species and play central roles in physiology. The prominent expression of P-gp and MRP1 in the human lung [4] suggests that these transporters may be pivotal in the protection against endogenous or exogenous toxic compounds entering the lung. The delivery of pulmonary drugs to reach the site of action may also depend on the presence and activity of many ABC transporters [5]. Langmann *et al.* developed quantitative real-time RT-PCR expression profiling of 47 ABC transporters in 20 different human tissues. Tissues with

a barrier function such as lung and trachea were identified to have high transcriptional activity for many ABC transporters [6]. There are already clear proofs of important functions of ABC transporters in the lung. The ABC transporter most widely investigated is the cystic fibrosis transmembrane conductance regulator (CFTR), because mutations in this gene are responsible for the development of cystic fibrosis [7]. Mutations in *ABCA1* are causative for Tangier disease [8]. In newborns it was shown that mutations in *ABCA3* cause aberrant production of surfactant which can be lethal [9]. The role of altered function of ABC transporters in highly prevalent pulmonary diseases such as asthma or chronic obstructive pulmonary disease (COPD) have hardly been investigated so far [10].

This review aims to give new insights in the possible functions of ABC molecules in the lung in view of their expression in different cell types. Furthermore, their roles in protection against noxious compounds will be discussed as well as the (mal)function in normal and pathological lung. We especially focused on the members of the ABCC subfamily of transporters (to which the MRPs and CFTR belong), the ABCB subfamily (a.o. MDR1/Pgp and MDR3/Pgp) and the ABCA subfamily (a.o. *ABCA1* and *ABCA3*), because these are a selection of the best characterised human transporters and because they have been investigated in human lungs. Results of *in vitro* studies in pulmonary research are being reviewed and an update will be given about what is known about pulmonary functional changes in ABC transporter knock-out mice models. In addition, the (potential) effects of polymorphisms in ABC transporters on lung functioning are discussed.

MDR1/P-gp

MDR1 localisation and function

The *MDR1* (ABCB1) gene is located on chromosome 7q21.12 and encodes for P-gp. P-gp plays a role in cell defence against environmental attacks such as generated by xenobiotics. It transports many hydrophobic substrates and anti-cancer drugs including etoposide, doxorubicin and vinblastine (for review, see [11]) and is mainly apically expressed in organs involved in excretion such as liver and intestine. In the lung, P-gp is expressed at the apical side of ciliated epithelial cells or ciliated collecting ducts, and on apical and lateral surfaces of serous cells of bronchial glands but not in mucus-secreting goblet cells (Figure 1 and 2)(Table 1) [12]. Epithelial cells of the trachea and major bronchi stain strongly while staining of the smaller bronchi is patchy or absent. In addition, P-gp is present in the lateral

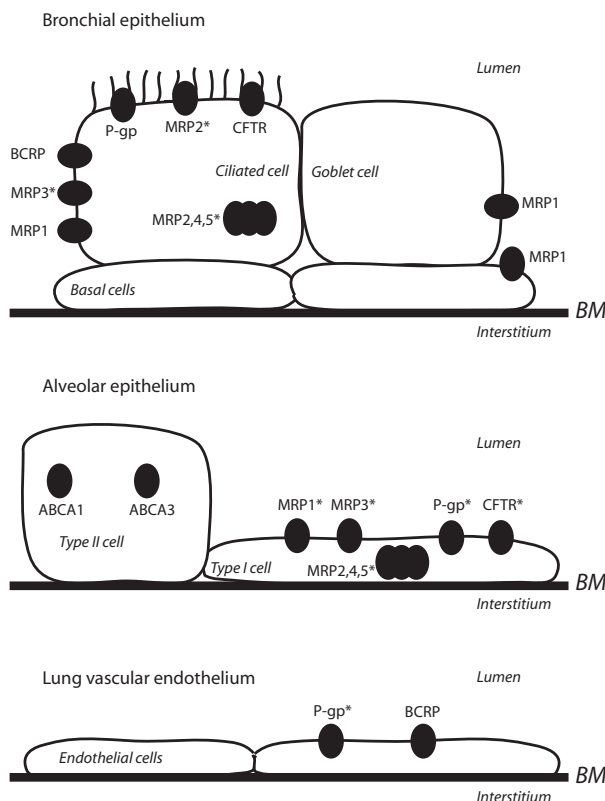


Figure 1. Expression of ATP-binding cassette proteins in several cell types of human lung. ABC, ATP-binding cassette; BM, basement membrane; BCRP, breast cancer resistance protein; CFTR, cystic fibrosis transmembrane

GSH, glutathione; *GSSG*, oxidized glutathione; *GS-X*, GS-conjugate; *ATP*, adenosine triphosphate;

membranes of normal nasal respiratory mucosa [13]. In human and rat type I alveolar epithelium, P-gp is located at the luminal side whereas freshly isolated type II cells lack P-gp [14]. In another study, pneumocytes did not stain for P-gp [15]. Some antibodies visualised P-gp in endothelial cells of blood vessels (Figure 1) [12, 15, 16]. In addition, alveolar and peripheral blood monocyte-derived macrophages stained positive but variably for P-gp [4, 16, 17]. The observed apical epithelial expression may signify that P-gp plays a role in transport of compounds from the interstitium into the lumen. However, the precise function of P-gp in the lung is as yet unknown. Interestingly, P-gp was found to play a role in the regulation of cell volume activated chloride channels and to possess channel activities [18, 19]. However, Cl^- and K^+ conductances were not affected by the level of P-gp

Table 1. Summary of features of ABC transporters in human lung

ABC gene	Gene location	Functional role or substrates	Linked to disease	Protein expression in lung cells (+ localisation[§])	References[#]
MDR1/P-gp (ABCB1)	7q21.12	Drug resistance Hydrophobic organic cations	Unknown	Bronchial epithelium (ap), mucinous glands (ap), alveolar type I cells* (ap), endothelium* (ap), alv. macroph.	[4,12,14-16]
MDR3/P-gp (ABCB4)	7q21.12	Phosphatidyl choline	Unknown	Absent	[4]
MRP1 (ABCC1)	16p13.12	Drug resistance Organic anions (e.g. GSH conjugates, LTC ₄)	COPD?	Bronchial epithelium (ba/la), goblet cells (ba), peripheral epithelial cells* (ba/la), seromucinous glands (ba/la), alv. macroph.	[4,53,85]
MRP2 (ABCC2)	10q24.2	Drug resistance Organic anions	Dubin-Johnson syndrome	Bronchial epithelium* (ap), primary bronchial and peripheral epithelial cells* (in)	[4,85,90]
MRP3 (ABCC3)	17q21.33	Drug resistance Organic anions	Unknown	Primary bronchial and peripheral epithelial cells* (ba/la)	[85]
MRP4 (ABCC4)	13q32.1	Nucleoside analogues, Prostaglandin E ₁ , E ₂	Unknown	Primary bronchial and peripheral epithelial cells* (in)	[85]
MRP5 (ABCC5)	3q27.1	Nucleoside analogues, Hyaluron	Unknown	Primary bronchial and peripheral epithelial cells* (in)	[85]
MRP6 (ABCC6)	16p13.12	Unknown	Pseudoxanthoma elasticum	Unknown	
MRP7 (ABCC10)	6p21.1	Drug resistance	Unknown	Unknown	
MRP8 (ABCC11)	16q12.1	Conjugated steroids, nucleoside analogues, bile acids	Unknown	Unknown	
MRP9 (ABCC12)	16q12.1	Unknown	Unknown	Unknown	
CFTR (ABCC7)	7q31.31	Chloride ion channel	Cystic fibrosis	Bronchial epithelium (ap), seromucinous glands (ap), Clara cells*, Alv. type I cells*	[117-120]
BCRP (ABCG2)	4q22	Drug resistance Protection food toxins	Unknown	Bronchial epithelium (ba/la), endothelium, seromucinous glands	[4]
ABCA1	9q31.1	Cholesterol and phospholipids	Tangier disease	Alv. type II cells	[151]
ABCA3	16p13.3	Surfactant secretion	Surfactant deficiency	Alv. type II cells	[155]

[§]Cellular localisation: ap, apical; ba, basal; la, lateral; in, intracellular; alv., alveolar; macroph., macrophages; [#]References are mentioned that demonstrate protein localisation histologically; *Conflicting results exist in literature.

expression and the physiological role of P-gp in volume-activated chloride currents is still unclear [20-22]. Many lipophilic amines, such as fentanyl, highly accumulate in the lung. It was suggested that P-gp plays a role in the disposition of these pulmonary amine drugs [23]. Cellular uptake in lung microvascular endothelial cells of fentanyl was inhibited by the P-gp substrate verapamil, but not by the P-gp blocking antibody UIC2. The authors suggested that this results from an inhibition of fentanyl uptake. This is however unlikely, since P-gp is primarily responsible for removal of drugs out of cells.

P-gp mRNA expression was not increased in smokers (n=11) compared to ex-and non-smokers (n=7) [12]. Whether P-gp expression levels may play a defensive role towards tobacco-derived agents remains to be investigated.

MDR1 in tumours

High P-gp expression can imply chemotherapeutic resistance due to increased chemotherapeutic drug efflux. In cancer therapy, many attempts have been made to reverse MDR mechanisms. However, in a randomised double-blind trial in 130 SCLC patients no positive effects were seen with the P-gp modulator megestrol acetate in addition to chemotherapeutic drugs, suggesting that levels of P-gp expression in lung tumours were not relevant or that modulation of P-gp activity was not complete in this treatment [24]. Some studies show higher P-gp expression at the invasion front of lung tumours and it was suggested that P-gp expression augments invasion properties of tumour cells [25]. Only two out of 22 NSCLC samples (both adenocarcinomas) stained positive with three P-gp antibodies [15] and no P-gp was detected on pulmonary carcinoids. Other studies revealed a relation between P-gp and glutathione S-transferase-pi (GST-pi) expression in NSCLC that were exposed *in vitro* to doxorubicin [26], suggesting that these two factors play a role in doxorubicin resistance. There was also a correlation between current smoking and doxorubicin resistance of NSCLC. Forty-two out of 72 NSCLC smokers expressed P-gp, whereas only two out of 22 tumours of non-smokers were P-gp positive [27].

MDR1 polymorphisms

MDR1 polymorphisms were first described by Hoffmeyer *et al.* [28] who found a correlation between lower intestinal expression of P-gp and a polymorphism in exon 26. Many single nucleotide polymorphisms (SNPs) have been recognised in the *MDR1* gene (see reference [29] for recent review about

clinical aspects). The impact of these polymorphisms on lung diseases is still speculative. It was proposed that polymorphisms in the *MDR1* gene may have clinical consequences in patients with cystic fibrosis, since MDR1 plays a role in CFTR regulation. Rodents contain two *Mdr1* genes, denoted as *Mdr1a* and *Mdr1b*. It was shown that *Mdr1b* mRNA expression in lung parenchyma of outbred rats is very variable and this may also be the case in humans [30]. The possible effects of *MDR1* polymorphisms was studied in tobacco-related lung cancer [31]. No clear association was found between the T/T genotype of the C3435T polymorphism and susceptibility to lung cancer in a group of 268 Caucasian men who were current smokers. No relation was found between SNP C3435T in *MDR1* and survival in 62 docetaxel-cisplatin-treated NSCLC patients [32]. Immunosuppressive agents such as cyclosporin A and tacrolimus (both calcineurin antagonists) are P-gp substrates. No relation was found of *MDR1* G2677T and C3435T genotypes with tacrolimus blood levels in 83 lung transplant patients treated with tacrolimus [33]. Altogether, these data implicate that there is still no clear association between *MDR1* polymorphisms and effects on outcome of treatment of lung cancer or lung transplant patients.

MDR1 in animal models

Scheffer *et al.* detected high P-gp levels in lungs of mice [4]. In rats, *Mdr1a* and *Mdr1b* mRNA expression were highest in the ileum [34]. The *Mdr1a* expression level in rat lung was 2% of the expression in ileum and expression of *Mdr1b* was 47% of that in ileum. In mice orally treated with dexamethasone for 24 hours, *Mdr1b* mRNA expression in lungs was decreased, from which the authors deduce that dexamethasone treatment of lung tumours may reverse MDR [35]. To study the *in vivo* distribution of P-gp, nude rats were injected with a P-gp overexpressing SCLC cell line (GLC4/Pgp) and with a P-gp negative cell line (GLC4) [36]. P-gp function was visualised with radiolabeled P-gp substrate [¹¹C]verapamil by positron emission tomography (PET) with or without P-gp modulator cyclosporin A. The accumulation of [¹¹C]verapamil was significantly increased by cyclosporin A in brains and GLC4/Pgp tumours in these rats. In all other investigated organs including lungs, the accumulation after cyclosporin A treatment was unaltered. In intact rabbit lung, vascular P-gp kinetics were measured *in vivo* using the lipophilic amine dye rhodamine 6G (R6G) by measuring R6G in the perfusate during circulation [37]. Inhibition of P-gp function with verapamil or GF120918 resulted in higher accumulation of R6G in lung.

It was proposed that the opposite would happen when epithelial P-gp was inhibited because R6G would then be retained in the airspace. We propose another possibility that inhibition of epithelial P-gp will also result in higher R6G accumulation. In that case, R6G transport to the lumen is inhibited and as a compensation mechanism it may be transported back to the interstitial side where it either may be retained in the tissue or subsequently be transported into the circulation. This model could be useful in testing a large variety of pulmonary therapeutic agents, such as corticosteroids and sympathicomimetics that may be substrates for transporters in the lung or modulate their activity. Similar studies were also carried out in perfused rat liver to assess the effect of P-gp modulators on the hepatobiliary system, supporting the usefulness of this approach [38].

Mdr1a/1b (-/-) mice lack both genes encoding for P-gp and these mice seem physiologically normal. The penetration of [³H]digoxin (a P-gp substrate) was higher in brain, ovary and adrenal gland. In the lung tissue of these (-/-) mice [39], the level of [³H]digoxin was rather low compared to other organs. The level was 2.6 times higher in (-/-) mice than in (+/+) mice, but this was not significant. The pharmacokinetics of the central nervous system (CNS) drug amitryptiline (a P-gp substrate) and its metabolites were examined in Mdr1a/1b (-/-) mice by high performance liquid chromatography (HPLC) in several organs. Higher concentrations of these metabolites were measured in the brain of these mice, but not in other examined organs including the lung [40]. Thus, P-gp likely plays a more active role in exporting CNS drugs out of the brain than out of the lung. Another possibility is that other transporters in the lung were capable to efflux these drugs as a compensation mechanism.

MDR1 in vitro

Several lung cell culture models have been described for determining P-gp expression and functionality. The immortalised human bronchial epithelial cell line 16HBE14o⁻ resembles primary epithelium and was reported to be suitable for drug metabolism studies [41]. In 16HBE14o⁻ cells, P-gp was expressed at the apical side. Its functional activity was measured with P-gp substrate rhodamine 123 and its transport was inhibited by verapamil [42]. Two lung cell lines, Calu-3 and A549 cells, were compared for P-gp expression and functionality [43]. The bronchiolar adenocarcinoma cell line Calu-3 is suitable for drug transport studies because these cells form tight junctions in culture, which is not the case for the type II alveolar carcinoma cell line A549. P-gp expression was higher in A549 cells than in Calu-3 cells.

However, efflux of rhodamine 123 was higher in Calu-3 cells. This may be explained by additional MRP1 activity in these cells because rhodamine 123 is also an MRP1 substrate [44]. In primary rat alveolar type II cells, Mdr1b mRNA levels increased in a time dependent manner in cultures at day 1, 2 and 3 compared to freshly isolated cells. Mdr1b mRNA was present at low levels and increased after oxygen radical induction with paraquat [45]. P-gp expression was below the detection limit in these cells at time of isolation. In freshly isolated primary human bronchial epithelial cells, P-gp was present and increased after 24 hours paraquat exposure. Rhodamine 123 efflux could be measured in these cells, confirming functional activity of P-gp [45]. These results demonstrate that P-gp is upregulated during stress, both from radical production and from *ex vivo* culturing or differentiation.

MDR3/P-gp

The MDR3/P-gp (ABCB4) gene maps closely to MDR1/P-gp on chromosome 7q21.12 and has high homology with MDR1 although its function is very different. It is involved in phosphatidyl choline transport from the liver into the bile. Its RNA and protein have not been detected in human and mouse lung and trachea [4, 6] and it is therefore not expected that MDR3 is present in the lungs.

MRP1

MRP1 localisation and function

The *MRP1* (ABCC1) gene is located on chromosome 16p13.12. Cole *et al.* discovered in 1992 a non-Pgp mediated MDR mechanism in the human lung cancer cell line H69AR [46]. MRP1 was overexpressed in these cells and it was found that glutathione-, glucuronide-, and sulfate-conjugated organic anions are substrates for MRP1 [47, 48]. MRP1 confers resistance to several chemotherapeutic agents including vincristine, daunorubicin and methotrexate [49-51]. Physiological substrates for MRP1 are e.g. leukotriene C₄ (LTC₄) and glutathione disulfide (GSSG) [52]. Interestingly, these substrates play an important role in lung physiology with respect to inflammation and oxidative stress. MRP1 is highly expressed mainly at the basolateral side of human bronchial epithelial cells (Figure 1 and 2) [53]. Ciliated and basal cells have been collected from brushes of main or lobar bronchi. Basal cells stained strongly on the entire circumference of the plasma membrane. Ciliated epithelial cells and mucous cells stained positive at the basolateral membrane but not at the apical membrane. No

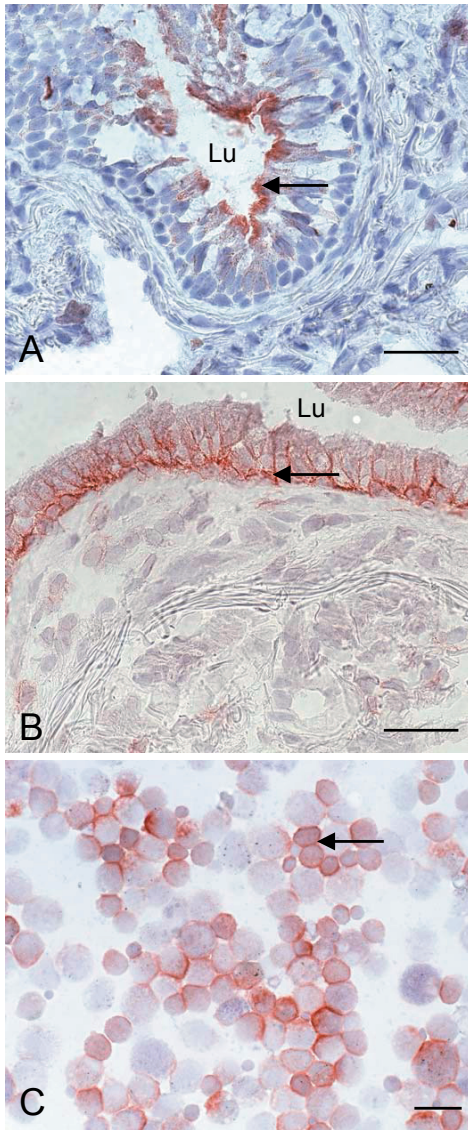


Figure 2. Immunohistochemical staining of ATP-binding cassette proteins in human lung. A. apical expression of P-gp in bronchial epithelium (COPD patient; frozen section, antibody C219), B. basolateral expression of MRP1 in bronchial epithelium (COPD patient; antibody MRPr1), C. MRP1 expression in bronchoalveolar lavage cells (healthy individual; antibody MRPr1). Lu, lumen. Scale bar = 25 μ M.

intracytoplasmic staining was observed. However, strong apical cytoplasmic staining has been detected below the cilia in respiratory columnar epithelial cells in paraffin sections with antibody MRPr1 [4, 13, 54]. The discrepancy between these findings may be due to different fixation procedures. Basal cells of seromucinous glands of the lungs also stain positive for MRP1 with a higher intensity in the serous area than mucinous cells. Alveolar macrophages are MRP1 positive with variable staining between individual lung samples (Figure 2) [4]. Kool *et al.* investigated MRP1, 2, 3, 4, and 5 with

an RNase protection assay in total lung RNA and found high MRP1, absence of MRP2, low MRP3 and MRP4 and moderate MRP5 gene transcripts [55]. Total RNA was obtained from lung tissue collected during surgery or autopsy. Therefore, the original cell types can not be distinguished in these samples. Possibly, the high MRP1 expression at the basolateral side of lung epithelium may assist in the clearance of toxins coming from the luminal or interstitial side (back) into the interstitial fluid [56]. Function of MRP1 expression in the lung epithelium, glands and alveolar macrophages may include extrusion of toxic intracellular substances, antioxidant defence or production of LTC₄ as an inflammatory response. Given the fact that MRP1 expression is higher in the lung compared to other solid organs, decreased or increased functional MRP1 expression may have a high impact on development and/or progression of lung diseases and protection against air pollution and inhaled toxic substances such as present in cigarette smoke [57].

MRP1 in tumours

Using mRNA *in situ* hybridisation Thomas *et al.* [58] detected high MRP1 expression in normal epithelium whereas the major component of the tumour epithelium showed a negative hybridisation signal. However, MRP1 transcript expression at the invasive front of the lung tumours was consistently stronger and particularly strong in areas with lymphatic or blood vessels. In addition, endothelial cells and lymphocytic infiltrates stained strongly positive for MRP1. These results may indicate a role of MRP1 in invasion or in mitotic activity. In a recent study, all 102 NSCLC tumours were MRP1 positive. In addition, the level of expression was 3-fold higher in DNA-aneuploid cells compared to normal bronchial and carcinomatous DNA-diploid cells [59]. This was associated with more frequent gain of chromosome 16 where the *MRP1* gene is located. Overexpression of MRP1 may therefore be an important factor of intrinsic resistance to chemotherapy in NSCLC. High MRP1 expression can also result in increased apoptosis as shown by experiments with verapamil in *MRP1* transfected baby hamster kidney-21 (BHK-21) cells [60]. Verapamil acts as an apoptogen in these cells when compared to MRP1 negative cells or mutant *MRP1* transfected control cells. This was accompanied by depletion of intracellular GSH due to transport of GSH by MRP1. Indeed, addition of extracellular GSH prevented cell death. This mechanism may be valuable for treatment of MRP1-positive tumours.

MRP1 in non-malignant diseases

Cigarette smoking is the principle risk factor for the development of COPD. Our preliminary results indicate that MRP1 expression is diminished in bronchial epithelium of COPD patients (ex-smokers) and that lower expression is related to worse lung function [57]. In addition, bronchial MRP1 expression was higher during smoking than after one year of smoking cessation (Van der Deen *et al.*, submitted). The expression of MRP1 measured with RT-PCR was similar in lungs of smokers and a combined group of ex- and non-smokers, suggesting that current smoking does not affect MRP1 gene expression [53]. This was semi-quantitatively analysed and the power of this study was rather low (smokers n=13 and ex- or non-smokers n=8). Cigarette smoke extract is a complex mixture of many substances. One of these is nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogenic nitrosamine. NNK is converted intracellularly in NNAL-O-glucuronide which is transported by MRP1 in the presence of glutathione (GSH) [61]. Thus, functional activity of MRP1 in the lung may play an important role in the antioxidant defence against toxic compounds generated by cigarette smoke.

MRP1 polymorphisms

Polymorphisms of the *MRP1* gene or in regulatory genes may influence function of MRP1 in the lung. *MRP1* has been screened for genetic variations and several mutations have been found in the *MRP1* gene in the human population [62, 63]. Ethnic differences for MRP1 expression were observed between Caucasian and Japanese subjects but the clinical consequences have to be determined to date [64, 65]. In preclinical models, a single mutation in the *MRP1* gene can result in loss of transport of some, but not all, MRP1 substrates [66] which implies that potentially minor differences in the *MRP1* gene may result in aberrant functional capacities of the MRP1 protein. A low frequency (<1%) naturally occurring mutation in *MRP1* was related to functional differences in organic anion transport and drug resistance [67]. Therefore, some individuals could be more susceptible to a set of xenobiotics than others.

MRP1 in animal models

Scheffer *et al.* detected, besides P-gp, high levels of Mrp1 in lungs of mice [4]. *Mrp1* (-/-) mice develop normally [68]. Viability, fertility, and a range of histological, hematological, and serum-chemical parameters were similar in

Mrp (+/+) and *Mrp* (-/-) mice. However, *Mrp1* (-/-) mice are hypersensitive to exposure to several drugs e.g. etoposide, resulting in loss of body weight and death [56, 68, 69]. In *Mrp1* deficient mice there were no gross abnormalities in lungs and other tissues after treatment with the chemotherapeutic drug etoposide-phosphate, but abnormal mucous production was seen around the mouth. Further examination of tissues showed that these mice suffered from oropharyngeal toxicity [56]. In *Mrp1* (-/-) mice, GSH levels were elevated in e.g. lung, kidney, heart, testes and skeletal muscle. In organs that express little MRP1, such as the liver and small intestine, GSH levels were unchanged [68, 70]. The increase in GSH was not related to increased levels of gamma-glutamylcysteine synthetase (gamma-GCS), the rate-limiting enzyme for production of the tripeptide GSH. This suggests that MRP1 plays a role in GSH metabolism because MRP1 expression determines cellular GSH levels which is gamma-GCS independent. In liver tissue, *Mrp2* and *Mrp5* mRNA levels were increased in *Mrp1*(-/-) mice compared to wild-type mice, probably due to a compensation mechanism. *Mdr1a* and *Mrp3* levels were unchanged in these animals. Unfortunately, lung tissue was not examined [70]. *Mrp1* (-/-) mice showed impaired inflammatory responses accompanied by a decreased LTC₄ excretion by leukotriene producing cells [68, 69]. The outgrowth of *Mycobacterium tuberculosis* was enhanced in *Mrp1* (-/-) mice, but there was no difference in survival compared to wild-type mice [71]. Strikingly, Schultz *et al.* [72] observed that the survival of *Mrp1* (-/-) mice inoculated with *Streptococcus pneumoniae* was better than of wild-type mice. This was accompanied by a lower LTC₄ concentration but a higher LTB₄ level in bronchoalveolar lavage fluid (BALF). Treatment with an LTB₄ antagonist abolished the positive effect on survival rate. Altering MRP1 function may therefore be a target for further studies on treatment of pneumonia. Interestingly, LTB₄ levels are also elevated in sputum and exhaled breath condensate of COPD patients [73, 74]. This is thought to result from a higher amount of neutrophils present, yet also lower functional MRP1 expression may play a role, according to the observations in *Mrp1* (-/-) mice [57, 72]. The pulmonary and hepatic carcinogen aflatoxin B₁ (a mycotoxin) and its GSH conjugate are MRP1 substrates *in vitro* [75]. MRP1 may therefore play an important role in detoxification of aflatoxin B₁ in the lung. No difference in occurrence of lung tumours (and liver tumours) 12 months after an 8 week exposure to aflatoxin B₁ was observed in *Mrp1* (-/-) and wild-type mice [76]. This observation may be explained by differences in exposure time

compared to humans (who may be chronically exposed for many years) or by redundancy of other ABC transporters in knock-out mice to export aflatoxin B₁ and its conjugates.

NRF2 (nuclear factor-E2 p45-related factor), a transcription factor for many genes that play a role in antioxidant defence and detoxification processes, was recently identified as a transcription factor for MRP1 [77]. Interestingly, the onset of cigarette smoke-induced emphysema was earlier and the extent of emphysema was more severe in *Nrf2* (-/-) mice as compared to wild-type mice [78]. The number of inflammatory cells, mainly consisting of macrophages, in bronchoalveolar lavage fluid and lung tissue was higher in *Nrf2* (-/-) mice, accompanied by more extensive apoptosis of endothelial cells and alveolar type II cells. Forty-five *Nrf2*-dependent protective genes were induced by smoke exposure in wild-type mice compared to knock-out mice as measured with microarray analysis. Regretfully, *Mrp1* was not present on this gene expression array. Similar smoke exposure experiments with *Mrp1* (-/-) mice would be interesting to investigate if these mice are also more susceptible to develop emphysema. Three polymorphisms were identified in the promotor region of *NRF2* in healthy individuals (n=81) and in patients with COPD (n=87) and sytemic lupus erythematosus(n=51) (all Japanese individuals) [79]. There was no relation between these polymorphisms and risk of these diseases, but the power of this study was rather low.

Mdr1a/1b(-/-)/*Mrp1*(-/-) triple knockout (TKO) mice are indistinguishable from wild-type mice under normal physiological conditions [80, 81].

Intravenous injection of [³H]etoposide in TKO mice resulted after 4 hours in a higher accumulation in brown adipose tissue, colon, urogenital tract, salivary gland and heart when compared to *Mdr1a/1b* (-/-) mice. The accumulation of [³H]etoposide in lung did not increase [80]. Accumulation of [³H]vincristine was 26-fold higher in lungs of weaning TKO mice compared to adult TKO mice, 4 hours after a single dose intraperitoneal injection [82]. In TKO compared to wild-type mice, [³H]vincristine was 82.7 and 5.8-fold increased in respectively weaning and adult mice after 4 hours in this experimental setting. Eight hours post-injection, these ratios were 33 and 51.1. Thus, accumulation of vincristine is both time and age-related. In another study, toxicity of the chemotherapeutic drugs vincristine and etoposide was elevated in TKO mice (respectively 128-fold and 3-5 fold) compared to wild-type mice. Lung tissue was not further examined [81].

MRP1 in vitro

MRP1 overexpression was first described in the human lung cancer cell line H69AR [46]. The human SCLC cell line GLC4/ADR, which displays multiple copies of the *MRP1* gene, is often used to study MRP1 function [47]. The lung cell line Calu-3 (from bronchiolar adenocarcinoma/glandular origin) has been reported to express MRP1 basolaterally and has functional MRP1 activity. It may serve as a good model for *in vitro* analysis of transport activities [44]. Besides P-gp, MRP1 protein was present in freshly obtained primary human bronchial epithelial cells. Efflux of MRP1 substrate carboxy-dichlorofluorescein could be measured in these cells and was inhibited by MRP modulator MK571 [45]. In contrast to upregulation of P-gp, epithelial cells did not respond with MRP1 upregulation to paraquat and MRP1 levels were stable in time until 12 weeks, but highly increased after 18 weeks culturing. An interesting observation was made by Bandi *et al.* [83] who incubated the airway epithelial cell line Calu-1 with budesonide, an anti-asthma drug. MRP1 expression decreased after 7 and 14 days incubation with budesonide (10 μ M) but not at day 1 and 4. MRP1 function was also diminished after 14 days incubation with increasing amounts of budesonide (1, 10 and 100 μ M). It was therefore proposed that budesonide could be used as a chemosensitiser in lung tumours. The immortalised bronchial epithelial cell line 16HBE14o⁻ expresses high MRP1 protein levels and activity [10]. MRP1 activity, but not P-gp, was blocked by incubation with cigarette smoke extract. Thus, the expression of MRP1 in human lungs may contribute to defence mechanisms of toxic inhaled substances present in cigarette smoke to protect against pulmonary diseases such as lung cancer or COPD. It was shown that benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon that is a.o. present in cigarette smoke and car exhaust fuels, increased the efflux of monochlorobimane GSH conjugate (mBCL-SG, which is an MRP substrate) out of primary rabbit alveolar type II cells [84]. This was explained by an increase in GSH levels. MK571 reduced the efflux of mBCL-SG, suggesting that the transport was MRP-mediated. Surprisingly, the transport direction of this substrate was higher to the apical than to the basolateral compartment. This may imply that MRP1 (or other MRPs) is apically located in alveolar type II cells in contrast to the basolateral location that is usually reported for MRP1, or that another transporter is responsible for this unexpected result. MRP1-5 expression was studied in primary human lung cell cultures and in A549 cells [85]. MRP1 and MRP3 expression were membrane-associated whereas MRP2, 4, and 5 were located in intracellular structures in primary

cells (both bronchial and peripheral epithelial cells). In A549 cells, all transporters were expressed in the cellular membrane. The fluorescent microscopic pictures, however, did not precisely show the exact cellular localisation and in several cases, the Golgi apparatus also seems to stain beside the cellular membrane.

MRP2

MRP2 localisation and function

MRP2 (ABCC2) is located on chromosome 10q24.2 and is also named the canalicular multispecific organic anion transporter (cMOAT) in the liver [86]. *MRP2* and *MRP1* share very similar substrate specificities. High affinity endogenous substrates for *MRP2* include amphiphilic anions, such as LTC₄ [87, 88] and bilirubin glucuronosides [89, 90]. Data on the presence of *MRP2* protein in the lung are conflicting. Only one out of three antibodies stained positive at the apical side in bronchial epithelium (Figure 1) [4, 90]. If *MRP2* is expressed apically, it could be involved in transport of noxious compounds into the lumen of the lung.

MRP2 in animal models

There are several rat strains that have a mutation in *Mrp2/cMOAT* and the phenotypes resemble that of the human Dubin-Johnson syndrome, a disease in which *MRP2* is mutated. Examples are the GY/TR⁻ rat [91] and the Eisai hyperbilirubinemic rat (EHBR) [92]. Subjects with the Dubin-Johnson syndrome and also *Mrp2* deficient rats display jaundice and have impaired organic anion transport. Recently, it was shown that deficient *Mrp2* function in EHBR rats was compensated by upregulation of *Mrp3* in liver and kidney [93]. *Mrp2* (-/-) mice have not been described. There are no reports in the literature of pulmonary malfunction in rats or humans with defects in the *MRP2* gene.

MRP3

The *MRP3* (ABCC3) gene is the closest *MRP1* homologue [94] and is located on chromosome 17q21.33. It is involved in resistance against the anti-cancer drugs etoposide, teniposide and at higher concentrations also to methotrexate [95]. *MRP3* is located in basolateral plasma membranes of liver, adrenals, pancreas, kidney, gut and gallbladder. The physiological function of *MRP3* is still unknown but its cellular localisation and the information on substrates implies a role for *MRP3* in transport of organic anions from

the liver into the blood, especially when secretion into bile is being blocked [96]. MRP3 protein and RNA was not detected in bronchial epithelium [4, 96]. However, data in literature are conflicting and preliminary unpublished results indicate that Mrp3 is present in mouse lung. As already mentioned in the discussion on MRP1, primary epithelial cells (of bronchial and peripheral origin) and A549 cells stain positive for MRP3 (Figure 1) [85]. MRP3 mRNA was measured by quantitative RT-PCR in normal lung and lung tumours. MRP3 transcript levels were related to exposure to vincristine, etoposide and platinum drugs in lung tumours [97, 98].

MRP4 and MRP5

MRP4 (ABCC4) and *MRP5* (ABCC5) genes map to 13q32.1 and 3q27.1 respectively. MRP4 protein is highly expressed in the kidney and prostate and MRP4 mRNA is present in the lung [55, 99]. MRP5 mRNA is ubiquitously expressed, mainly in brain and skeletal muscle and also in the lung [6, 55]. Primary epithelial cells (of bronchial and peripheral origin) and A549 cells stain positive for MRP4 and 5 (Figure 1) [85]. The substrate specificities of these two transporters differ from the other transporters of the ABCC family, i.e. they transport a variety of nucleoside analogues. The physiological role of both proteins is still unknown but they can serve as an efflux pump of the nucleosides cAMP and cGMP at low affinity, likely in a GSH independent manner [100-103]. Interestingly, AMP levels in lungs of asthma and COPD patients are elevated and therefore, MRP4 and MRP5 activity may be of clinical relevance in these diseases [104]. MRP4 can actively efflux prostaglandins E_1 and E_2 [105]. It was also suggested that MRP4 plays a role in the transport of conjugated steroids and bile acids [106], whereas MRP5 was reported to transport hyaluran out of cells [107]. In a recent study, a panel of 60 human cancer cell lines (the NCI-60) were screened with real-time RT-PCR for 48 human ABC transporters. Among these were several lung cancer cell lines. An association was found between MRP4 and MRP5 gene expression and resistance against platinum drugs in lung cancer [108-110].

MRP6

MRP6 (ABCC6) maps to chromosome 16p13.12 and is mainly expressed in liver and kidney. MRP6 was neither detected in the lung nor in lung derived tumour cell lines SW1573 and GLC4 by immunohistochemistry [111]. However, MRP6 mRNA was moderately present in human lung extracts [6]. MRP6 is mutated in the hereditary connective tissue disorder

pseudoxanthoma elasticum which affects skin, retina and blood vessels (for review, see [112]). Still, its physiological role in this disease and its substrate specificity is unclear. Pulmonary abnormalities are rare in pseudoxanthoma elasticum. However, in some patients calcification and elastic tissue damage in the lung have been described [113]. This may be due to an altered MRP6 function. Further studies are required to assess whether dysfunction of MRP6 also plays a role in development of other lung diseases such as emphysema. With *in situ* hybridisation and RNase protection assay in C57BL/6 mice, MRP6 mRNA could be detected in tracheal and bronchial epithelium [114].

MRP7, MRP8 and MRP9

To date, the functions of MRP7 (ABCC10, gene 6p21.1), MRP8 (ABCC11, gene 16q12.1) and MRP9 (ABCC12, gene 16q12.1) are largely unknown. Of these recently discovered ABC transporters, only MRP7 mRNA is highly expressed in total lung and trachea RNA extracts [6]. MRP7 function resembles P-gp function in the resistance against taxanes [115]. MRP8 resembles MRP4 function more than MRP5, and the physiological role of MRP8 may involve transport of conjugated steroids, cyclic nucleotides and bile acids [116].

CFTR

CFTR localisation and function

CFTR (ABCC7) is located on chromosome 7q31.31. CFTR is the only member of the ABC superfamily which is not an active transporter. It functions as a chloride channel and in normal human airway tissue CFTR is highly expressed at the luminal side in serous cells of the submucosal glands. In addition, it is restricted to the apical membrane domain of well-differentiated epithelial cells such as ciliated cells, and probably also non-ciliated Clara cells and alveolar type I cells (Figure 1) [117-120]. CFTR is also expressed in normal nasal respiratory mucosa [13].

CFTR in cystic fibrosis

Mutations in the CFTR gene can cause cystic fibrosis [7] and are associated with abnormal Cl⁻ and Na⁺ ion transport in several tissues including the lungs, pancreas, gastrointestinal tract, liver, sweat glands and male reproductive organs. Although the normal expression of CFTR in the lung is lower compared to tissues such as the intestine and pancreas, its function in the lung is of major importance. The most frequent mutation in this

gene is the delta F508 mutation which leads to cystic fibrosis. Defective CFTR function can cause viscous secretions in the lungs which leads to chronic inflammation with acute exacerbations by impaired mucociliary clearance. There are major risks for colonisation with *Pseudomonas aeruginosa* which leads to pneumonia and respiratory insufficiency [121]. Besides abnormal CFTR localisation and expression in cystic fibrosis, also in non-cystic fibrosis airway tissue CFTR can be abnormally expressed in remodelled or dedifferentiated epithelium [122, 123] whereas in delta F508 CFTR epithelial cells there may be a normally processed CFTR [120, 124]. The regulation and transport function of CFTR are dependent of the state of differentiation and polarisation of epithelial cell cultures [125, 126]. Dedifferentiation with hyperplasia or metaplasia was associated with an intracellular localisation or absence of CFTR protein [122]. Hurbain *et al.* [127] analysed MRP1-5 transcript levels in nasal respiratory cells from cystic fibrosis patients with homozygous delta F508 mutation. Surprisingly, low MRP1 levels were associated with more severe disease and in addition, MRP1 levels were related to cAMP-independent chloride transport suggesting that MRP1 regulates another chloride channel in the apical membrane. CFTR function is cAMP regulated [121], and therefore, MRP4 or MRP5 activity may play a role in regulation of CFTR, since these proteins transport cAMP. Another study showed that CFTR function is blocked by two MRP substrates (tauroolithocholate-3-sulphate and beta-estradiol) [128]. This implies that these two substances are also substrates for CFTR, or that CFTR and MRP proteins possess similar anion binding sites. Bebok *et al.* showed that nitric oxide (NO) and reactive oxygen nitrogen species (RONS) decrease wild-type CFTR protein levels in airway epithelial cell monolayers [129]. Natural sources of NO and RONS are activated in alveolar and interstitial macrophages [130, 131], neutrophils [132], alveolar type II cells [133, 134] and airway epithelial cells [135]. In this view, CFTR function may be important in pulmonary infections and in the effect of oxidative stress generated by e.g. cigarette smoke.

CFTR in animal models

Cftr knock-out mice show reduced viability in contrast to most other ABC transporter knock-outs which are viable and fertile. Trezise *et al.* developed *Cftr* knock-out mice that have a severe cystic fibrosis phenotype accompanied by a lack of *Cftr*-related chloride conductance in e.g. tracheal epithelium [136, 137]. They found that the P-gp mRNA level was four-fold

increased in intestines of neonatal and 3- to 4-week-old *Cftr* knock-out mice compared to littermates of the same age. However, in 10 weeks-old mice P-gp levels were three-fold decreased. Apparently, a reduction or loss of *Cftr* function influences P-gp expression.

CFTR in vitro

The Calu-3 cell line, which has properties from serous cells of the pulmonary submucosal glands, is often used to study function and expression of CFTR [129, 138]. It was demonstrated in Calu-3 cells that CFTR mRNA expression is downregulated after ouabain incubation whereas P-gp expression was upregulated [139]. The bronchial epithelial cell line 16HBE14o⁺ is also invaluable in CFTR research [129, 140]. In wild-type and mutant CFTR expressing Sf9 insect cells, it was demonstrated that besides chloride transport, another function of CFTR is transport of GSH in a nucleotide-dependent manner [141]. This observation suggests that CFTR plays a role in the control of oxidative stress. Indeed, several studies in patients, mice and cell lines have shown that GSH levels are lower in case of defective CFTR function.

BCRP

BCRP localisation and function

BCRP (ABCG2) is the breast cancer resistance protein (BCRP), located on chromosome 4q22. BCRP is a half-transporter that probably acts as a homo- or heterodimer [142] and is involved in resistance against toxins and several chemotherapeutic agents (e.g. mitoxantrone and topoisomerase 1 inhibitors) [143, 144]. Protein levels of BCRP in the lung are lower than P-gp and MRP1 but distinct in the epithelial cell layer and in seromucinous glands (Figure 1) [4]. BCRP was absent in alveolar macrophages, suggesting that BCRP does not play a major role in innate inflammatory responses in the lung. Small endothelial capillaries also stain positive for BCRP, thus BCRP may protect the lungs against noxious compounds that enter systemically.

BCRP in tumours

In a study of untreated solid lung tumours, i.e. squamous cell carcinoma (n=5), adenocarcinoma (n=2) and SCLC (n=3), most cases expressed moderate or strong BCRP [145]. In addition, higher BCRP expression in blood vessels was observed than in vessels of the surrounding tumour, indicating that BCRP may play a role in tumour angiogenesis. In 72 cases of advanced

NSCLC, BCRP expression, but not P-gp, MRP1, MRP2 and MRP3, predicted poor clinical outcome [146].

BCRP in animal models

Bcrp knock-out mice do not display any abnormalities compared to wild-type mice under normal conditions, except for the colour of their bile which is red instead of yellow on certain diets [147]. However, these mice appear to be extremely sensitive to the phototoxin pheophorbide A, showing that BCRP is important to protect against toxic food components. Abnormalities of lungs in *Bcrp* (-/-) mice have not been reported thus far. Recently, *Bcrp1* (*Bcrp* in the mouse is also called *Bcrp1*) localisation in the lung of 4- to 8-week old C57Bl/6J mice was studied to identify lung stem cells. These cells possess high activity of efflux of Hoechst dye that is transported by *Bcrp1* [148].

In peripheral blood and bone marrow, cells with side population activity represent BCRP positive (haematopoietic) stem cells and this might also be the case for side population cells in the lungs. Indeed, *Bcrp1* (-/-) mice did not display side population cells as shown by Hoechst staining of lung digests.

In paraffin-embedded tissue sections, *Bcrp1* was restricted to smooth muscle cells (of both arteries and airways) and a subpopulation of unidentified round cells in the alveolar space but not detectable in endothelial or epithelial cells.

BAL cells of mice were *Bcrp1* positive, but did not display side population (SP) activity [148]. Clara cells, a nonciliated bronchiolar epithelial cell type, were also phenotypically described to possess stem cell characteristics [149]. Further investigation is required to identify the stem cell pool in the lung in relation to expression of ABC transporters. Repairing and replacing lost lung tissue is a research area of promising therapeutic possibilities [150].

ABCA1

The *ABCA1* gene is mapped to chromosome 9q31.1. It is the causative gene in the development of Tangier disease, a disorder of cholesterol transport [8]. The ABCA1 protein controls transport of cholesterol and phospholipids to apolipoprotein 1 (apoA-1) in alveolar type II cells (Figure 1). Using the siRNA technique for ABCA1, it was shown that ABCA1 is involved in basolateral transport of surfactant that is activated by oxysterol [151]. *Abca1* knock-out mice show increased concentrations of cholesterol precursors in lung, plasma, intestine and faeces [152]. It was demonstrated that there were major morphologic abnormalities in lungs of these mice, increasing with age. In 30% of 18 months old mice, lung parenchyma was affected. Lesions were

characterised by foamy type II pneumocytes with aberrant lamellar bodies, intraalveolar macrophages and cholesterol clefts [153]. In addition, ABCA7, a close homologue of ABCA1 was also found to be highly expressed in mouse lung tissue by Western blot analysis [154].

ABCA3

The *ABCA3* gene is located on chromosome 16p13.3. The function of this ABC transporter has not been studied in detail but ABCA3 protein is present in lamellar bodies in human lung alveolar type II cells (Figure 1). Langmann *et al.* performed quantitative RT-PCR in 20 human tissues and found ABCA3 to be expressed restrictively in the lung [6]. In a recent study in cell lines, an association was found between lung cancer and ABCA3 (and also ABCA2) gene expression by means of quantitative RT-PCR [108].

The high expression of ABCA3 in alveolar type II cells suggests that ABCA3 may play a role in surfactant regulation [155]. Surfactant is important to lower the surface tension in the air-liquid interphase in alveoli. Indeed, in patients with surfactant deficiency and with severe neonatal lung disease it was demonstrated that the *ABCA3* gene was frequently mutated [9]. *ABCA3* mutations were found in 16 out of 21 patients, but polymorphisms (SNPs) were not found. One mutation was not fatal but was associated with a chronic lung disorder in a 6-year old patient. Other mutations turned out to be fatal and these patients died shortly after birth. Electron micrographs of tissue of patients with surfactant deficiency showed abnormal dense and small lamellar bodies. It was suggested that ABCA3 plays a role in phospholipid metabolism, since it closely resembles ABCA1 and ABCA4 that are known to transport phospholipids.

Conclusion

Little is known about the function of ABC transporters that are expressed in the lung although their overall expression is very high compared to many other organs [6]. This review shows that ABC transporters in the lung are not only relevant for relatively rare diseases such as cystic fibrosis, Tangier disease and surfactant deficiency. Preliminary data indicate that MRP1 expression is lower in COPD patients than in healthy controls. Mutations and polymorphisms in ABC transporters may have important clinical consequences for development of lung diseases. However, overlap in substrate specificities may be compensatory in cases of malfunction of one

(or more) transporter(s). Given the complexity of lung architecture, research on detailed cellular processes is difficult but challenging. Several ABC transporter deficient animal models have been developed that are of great value to study the role of these proteins. To date, exposure to cigarette smoke has never been tested in ABC transporter deficient animal models and would potentially give interesting information about the role of ABC transporters in protection against inhaled toxic substances such as present in tobacco smoke. Cell line models have been used to study transport processes and pulmonary drug metabolism. The delivery of pulmonary drugs to the site of action is probably highly dependent on the presence and activity of many ABC transporters in several cell types in the lung. The first barrier after inhalation is the pulmonary epithelium and transporters in the pulmonary endothelium may be critical for the delivery of intravenously or orally administered drugs. Insight in the function of ABC transporters in the lung may open new ways to facilitate treatment of lung diseases.

Abbreviations

ABC = ATP-binding cassette

AMP = adenosine monophosphate

BALF = bronchoalveolar lavage fluid

B[a]P = benzo[a]pyrene

BCRP = breast cancer resistance protein

cAMP = cyclic adenosine monophosphate

CFTR = cystic fibrosis transmembrane conductance regulator

cGMP = cyclic guanosine monophosphate

cMOAT = canalicular multispecific organic anion transporter

CNS = central nervous system

COPD = chronic obstructive pulmonary disease

EHBR = Eisai hyperbilirubinemic rat

gamma-GCS = gamma-glutamylcysteine synthetase

GSH = glutathione, reduced form

GSSG = glutathione disulfide; oxidized glutathione

GST = glutathione S-transferase

HPLC = high liquid performance chromatography

LTC₄ = leukotriene C₄

LTB₄ = leukotriene B₄

MDR = multidrug resistance

MRP = multidrug resistance-associated protein

NSCLC = non-small cell lung cancer

NNAL = nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

NNK = nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NO = nitric oxide

NRF2 = nuclear factor-E2 p45-related factor

P-gp = P-glycoprotein

R6G = rhodamine 6G

RONS = reactive oxygen nitrogen species

RT-PCR = reverse transcriptase-polymerase chain reaction

SCLC = small cell lung cancer

SNP = single nucleotide polymorphism

TKO = triple knock-out mice

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